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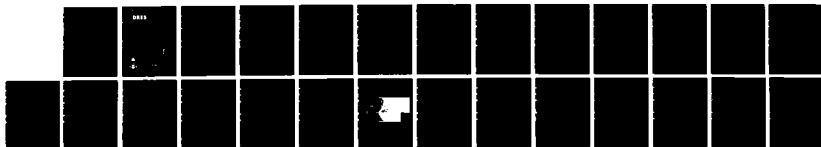
HIGH TEMPERATURE INDUCED ANTIBIOTIC SENSITIVITY IN
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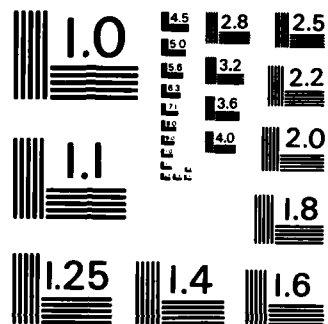
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HIGH TEMPERATURE INDUCED ANTIBIOTIC SENSITIVITY IN *PSEUDOMONAS AERUGINOSA* (U)

by

A.R. Bhatti*, K. Kumar†, C. Stobo† and J.M. Ingram†

Project No. 16A10

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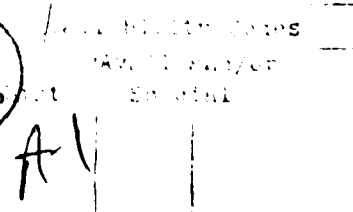
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**HIGH TEMPERATURE INDUCED ANTIBIOTIC SENSITIVITY
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INTRODUCTION

Pseudomonas aeruginosa (ATCC 9027) secretes periplasm-located proteins during growth (6) and these proteins are also liberated from whole cells after suspension in 0.2 M MgCl₂ (2). Cells suspended in Mg²⁺ are susceptible to the action of lysozyme as evidenced by spheroplast formation. This suggests that the permeability barrier of the outer cell wall is altered such that a molecule of molecular weight 14,000 daltons (lysozyme) is able to penetrate to the mucopeptide layer. Further testing of this hypothesis with actinomycin-D (mol. wt. 1200 d) suggested that this inhibitor of DNA-dependent RNA polymerase does not permeate the cytoplasm under these conditions (3). Treatment with ethylenediamine-tetraacetic acid (EDTA) was required for the actinomycin-D directed inhibition of RNA synthesis in whole cells. These data suggest that EDTA is necessary to alter the permeability of the cytoplasmic membrane and that this structure is the determinant permeability barrier within the bacteria cell.

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P. aeruginosa forms filaments when cultured at 46°C (1) and under these conditions the cells fail to divide; they do, however, increase in mass. These cells continuously secrete periplasm-located monomers of alkaline phosphatase (APase) (1) which are inactivated at the elevated temperature. Alkaline phosphatase will form a complex with lipopolysaccharide (LPS) and the complexed enzyme exhibits differences in reaction kinetics and substrate specificity when compared to the purified enzyme (4). These data indicate that the APase of *P. aeruginosa* is secreted *in vivo* as an APase-LPS complex.

During a study of the effects of high temperature on growth, it was shown that filaments were sensitive to low levels (25 µg/mL) of penicillin-G (7) whereas cells grown at 37°C remained resistant. The sensitivity of cells cultured at 46°C to other antibiotics was examined and it was shown that these cells are generally more susceptible to antibiotic action. This increased sensitivity to antibiotics is demonstrated by the present study to be due to a selective alteration in the permeability of the outer wall membrane because of the decreased LPS content.

MATERIALS AND METHODS

Organism and Culture Conditions

P. aeruginosa ATCC 9027 was maintained on Pseudomonas P agar slants (Difco Laboratories, Detroit, MI.). The organism was cultivated at 37°C or 46°C in a proteose peptone (Difco) — glucose — ammonium salts — tris (hydroxymethyl) amino methane (Tris) liquid medium as previously described (2).

Cultures were inoculated with a 1.0% inoculum grown under conditions as described above. The flasks were incubated in a Psychotherm shaker (New Brunswick Scientific, New Brunswick, NJ.) at the indicated temperatures. The determination of the release of periplasm-located APase and the preparation of Mg²⁺ spheroplasts were as described previously (2). Protein was determined by the method of Lowry *et al.* (9) and lipopolysaccharide (LPS) was determined as 2-keto-3-deoxyoctulonic acid (KDO) reactable material by the method of Osborn (14) after extraction by the procedure of Westphal *et al.* (17). Alkaline phosphatase activity was determined with p-nitrophenyl-phosphate (pNPP) as the substrate (2). Tris, lysozyme, pNPP, streptomycin and chloramphenicol were obtained from the Sigma Chemical Co., St. Louis, MO. Radioactive materials were obtained from New England Nuclear Ltd., Boston, Mass. Penicillin-G was obtained from Ayerst Laboratories, Montreal, Que.

Sensitivity to Antibiotics on Solid Media

Cultures of *P. aeruginosa*, grown for 8 to 10 h at 37 and 46°C, were diluted and aliquots spread on Pseudomonas-P agar plates. After 30 min incubation at the respective temperatures, 10 sensi-discs (Difco Laboratories, Detroit, MI.) of different antibiotics were aseptically applied to the surface. Duplicate plates were incubated at 37°C or 46°C. The diameters of the zones of inhibition were measured after 24 hours.

Sensitivity to Antibiotics in Liquid Media

P. aeruginosa was grown at 37 or 46°C for 4 to 6 h and filter sterilized antibiotics was added aseptically to give the final desired concentrations. The cultures were incubated at the two temperatures, aliquots withdrawn and analyzed for growth at 660 nm (Gilford model 300-N spectrophotometer). Samples were also examined by phase contrast microscopy for changes in general cell morphology.

Sensitivity of Spheroplasts to Actinomycin-D

The sensitivity of spheroplasts to actinomycin-D (a generous gift from Merck, Sharpe and Dohme, Pte. Claire, Que.) was assayed by determining the inhibition of ribonucleic acid synthesis (3). *P. aeruginosa* was grown at 46°C to an optical density of 0.55 at 660 nm. Magnesium-lysozyme spheroplasts were prepared as previously described (3), suspended to a final optical density of 1.0 at 660 nm and divided into three equal portions of 10 mL in 50 mL flasks. Actinomycin-D (20 µg/mL) was added to one flask, to a second actinomycin-D and EDTA (10 mM final concentration) and the third flask served as a control. Flasks were preincubated at 46°C for 5 min and uracil-2-¹⁴C (spec. act. 56.6 mCi/mM) was added to each flask. Samples (0.2 mL) were removed at 5 min intervals and diluted into 5.0 mL of ice cold trichloro-acetic acid (TCA, 10% w /v) maintained at 4°C for 16 h and filtered through a Millipore filter (0.45 µ, Millipore Filter Corp., Boston, Mass.). The precipitates were washed twice with TCA and dried in scintillation vials. Ten mL of toluene liquid scintillation fluid was added and the radioactivity determined with a Nuclear Chicago Isocap 300 liquid scintillation spectrometer.

RESULTS

Susceptibility to Antibiotics

Cell wall mutants of some bacteria exhibit increased sensitivity to antibiotics (12, 13). The sensitivity of *P. aeruginosa* to several antibiotics under various conditions was tested and the results are presented in Table I. The sensitivity of cells grown at 37°C to antibiotics which affect protein synthesis (chlorotetracycline and tetracycline) was greater at 46°C than at 37°C. *P. aeruginosa* was sensitive to kanamycin, streptomycin and chloramphenicol only at 46°C whereas polymyxin-B was equally inhibitory at both temperatures. *P. aeruginosa* grown at 37°C is resistant at either temperature to antibiotics such as penicillin-G and ampicillin which affect cell wall synthesis.

The sensitivity of *P. aeruginosa* filaments obtained after growth at 46°C and treated at 46°C or 37°C is shown in the right hand column of Table I. The organism is more sensitive to tetracycline, chloramphenicol, chlorotetracycline and kanamycin at 46°C. Polymyxin-B is equally effective at 46 or 37°C. Filaments obtained at 46°C are more sensitive to penicillin-G, ampicillin, erythromycin and lincomycin. Antibiotics which are effective against cell wall synthesis exhibit only slight inhibition (H) in cells grown at 46°C. Secondary, resistant, colonies are observed within the zone of inhibition after 48 h of incubation at either temperature. These resistant colonies are observed more frequently in filaments obtained after growth at 46°C and incubated at either 46 or 37°C in the presence of chlorotetracycline, chloramphenicol, streptomycin, tetracycline and polymyxin-B. Colonies resistant to chloramphenicol were observed only when the cells were grown at 37°C and incubated at 46°C. The results shown that cells of *P. aeruginosa* grown at 46°C are sensitive to antibiotics irrespective of the temperature of incubation and that the cell walls of 46°C grown cells are more "permeable" to these antibiotics.

Effect of Penicillin-G on the Growth and Cellular Morphology of *P. aeruginosa*

The data of the previous section suggested that permeability changes occurred in the outer cell wall of *P. aeruginosa* grown at 46°C. This hypothesis was tested by growing the organism at 37 and 46°C in the presence and absence of two concentrations of penicillin-G (25 µg and 100 µg/mL). The antibiotic had no effect on growth at 37°C (Figure 1) and phase contrast microscopy indicated that the cells were normal in all respects 3 h after the addition of penicillin. Cultures grown at 46°C, however, were

severely inhibited by both concentrations of penicillin (Figure 1) and cells in the presence of 25 $\mu\text{g}/\text{mL}$ of penicillin showed a "ballooning" effect at regular intervals (at the sites of constriction) and the distal ends (Figure 2). The decreased absorbance of cultures containing 100 $\mu\text{g}/\text{mL}$ penicillin is due to lysis of the filaments. These results substantiate the view that cells grown and treated at 46°C are more permeable to penicillin and that the "ballooning" most probably occurs at sites of active mucopeptide synthesis, i.e., the potential regions of filament division.

Streptomycin and Growth of *P. aeruginosa*

Streptomycin at 25, 50, 75 and 125 $\mu\text{g}/\text{mL}$ was added to cultures growing at 37 and 46°C and the results obtained are shown in Figure 3. Cells grown at 46°C showed sensitivity to all concentrations of streptomycin (only 25 and 125 $\mu\text{g}/\text{mL}$ shown). Lower sensitivity was observed at 37°C but *P. aeruginosa* was sensitive to increasing concentrations of the drug.

Actinomycin-D Effects on Cells and Spheroplasts

Cultures were grown at 37 and 46°C, treated with actinomycin-D and the results are presented in Figure 4. This inhibitor of DNA-dependent RNA synthesis has little or no effect upon *P. aeruginosa* under these conditions. The permeability changes which occur in the outer cell membrane are not sufficient to permit the penetration of actinomycin-D to its site of action in the cytoplasm. Although it was shown in previous experiments that growth at 46°C induced permeability changes in the outer membrane, it was desirable to determine whether or not alterations in the cytoplasmic membrane occurred at the elevated temperature. The results of Figure 5 show that the rate of uracil-2-¹⁴C incorporation into the nucleic acid of spheroplasts is only slightly inhibited at 20 $\mu\text{g}/\text{mL}$ of the antibiotic. The incorporation of uracil was completely inhibited in the presence of EDTA (10 mM) and actinomycin-D. These results are similar to those previously reported by Cheng *et al.* (3) and support the contention that EDTA alters the permeability of the cytoplasmic membrane as well as the outer cell wall thereby facilitating the penetration of actinomycin-D to its site of action.

Inhibition of Alkaline Phosphatase Synthesis by Antibiotics

Alkaline phosphatase of *P. aeruginosa* is a periplasm-located enzyme whose synthesis is depressed in the absence of inorganic phosphate. The enzyme is synthesized

in the cytoplasm as a monomer and transported to the periplasm where it dimerizes and interacts with LPS (6). The actions of various inhibitors of protein synthesis were examined for their effects upon APase. The concentrations of chloramphenicol and streptomycin used in the experiment do not completely inhibit growth at 37°C. The organism was grown at 37°C until the quantity of cell bound APase reached 0.035 IU/mL. The culture was then divided into three portions and 25 µg/mL of chloramphenicol was added to one flask and 125 µg/mL of streptomycin to another. All flasks were further incubated at 37°C and sampled for APase activity as reported in Figure 6. Except for the first hour, there was no marked increase in growth in the presence of the antibiotics. In addition, there was no increase in APase in the first 30 min in the presence of either antibiotic whereas the activity increased linearly in the control. After 30 min in the presence of the antibiotics, the rate of synthesis of APase proceeded as in the control cells but the level of enzyme remained low. It is obvious from the data that the synthesis of new APase at 37°C ceased virtually immediately after the addition of either of these two antibiotics but that the effect of these antibiotics was transient.

The effect of EDTA and actinomycin-D upon the synthesis of APase was examined and the results reported in Figure 7A. Unlike APase synthesis in *E. coli* (18), EDTA treatment of *P. aeruginosa* not only causes a decrease in the efficiency of enzyme synthesis but it also interferes with the kinetics of the derepression process (curve 2). In the first 30 min, EDTA- and actinomycin-D-containing cultures showed the same response with regard to APase synthesis, i.e., additional APase synthesis was almost eliminated. During the later stages of growth, APase synthesis continued at a slow rate both in the presence of EDTA, alone, and EDTA + actinomycin-D; however, in the presence of EDTA + actinomycin-D there was less APase synthesized (curve 3). APase synthesis in the control culture proceeded in a linear fashion and the rate was proportionately greater than that observed in cultures which contained EDTA and actinomycin-D. After 0.5, 1, 2 and 3 h, the EDTA treated culture had 8-, 7-, 6- and 5-fold less APase activity as compared to the control (curve 1), while in the presence of EDTA + actinomycin-D, the cultures had 7.5-, 8-, 8- and 6-fold less APase activity, respectively. It was also noted that these inhibitors did not have a pronounced effect on the growth of *P. aeruginosa* at 37°C (Figure 7B).

Growth Temperature and Lipopolysaccharide Content of *P. aeruginosa*

Lipopolysaccharide, as KDO-containing material, was quantitated in cells after growth at 37 and 46°C (Table 2). The cells contained less LPS when grown at 46°C as compared to cells at 37°C. When the cell-free culture filtrate (CFC) was analyzed for sedimentable and non-sedimentable LPS, it was observed that there was more non-sedimentable LPS in the CFC of cells grown at 37°C and the CFC of cells grown at 46°C had more sedimentable LPS (Table 3). The data of Table 3 also shows that cells grown at 46°C excrete more LPS into the growth medium and that cells grown at 37°C produce more total LPS than cells grown at 46°C (3.37 µg/mg protein compared to 2.23 µg/mg protein).

DISCUSSION

Pseudomonas aeruginosa shows increased sensitivity to antibiotics such as chlorotetracycline, tetracycline, lincomycin and erythromycin when cultured and tested at 46°C. On the other hand, this organism exhibits sensitivity at either 37 or 46°C to polymyxin B, whereas the cell wall antibiotics penicillin-G and ampicillin are only effective when cells are grown at 46°C and tested at 46°C. If the cells are shifted to 37°C prior to the addition of penicillin-G or ampicillin, no lysis is observed and the cells recover (C. Stobo and J.M. Ingram, unpublished). Cells grown at 46°C form filaments and penicillin-G acts on these cells at points of cell division. This is reflected in the "ballooning" nature of the outer cell wall which is obviously increasing in mass while the synthesis of new mucopeptide is blocked by the antibiotic. If this process is allowed to proceed unimpeded then the cytoplasmic membrane, followed by the cytosol, will flow into the "balloon" area creating a large spheroplast which subsequently lyses. It is also apparent from these observations that a competition of rates exists following a temperature shift from 46 to 37°C. If cells are shifted to 37°C prior to penicillin-G addition, then the lesion in the outer cell wall is repaired at a rate faster than the rate of penicillin-G penetration to, and reaction with, the mucopeptide layer. If penicillin-G added prior to a shift from 46 to 37°C, the cells form spheroplasts and lyse, indicating that the penicillin action occurred before the repair of the lesion.

The effect of antibiotics which must penetrate the cytoplasmic membrane to express their inhibitory action follow a different pattern than the mucopeptide synthesis-inhibiting antibiotics. Cells grown at 46°C were more sensitive to streptomycin than cells

grown at 37°C. At 37°C, the sensitivity to this antibiotic was concentration dependent whereas at 46°C, growth ceased at the lowest concentration of antibiotic employed (25 µg/mL). Actinomycin-D, on the other hand, is not inhibitory to cells or spheroplasts obtained from cultures grown at 37 or 46°C. In either case, EDTA is required to alter the permeability of the cytoplasmic membrane. These results with filaments, and spheroplasts obtained from filaments of *P. aeruginosa* grown at 46°C, suggest that the outer cell wall serves as a primary barrier to molecules such as lysozyme (1) and actinomycin-D (3) but that this layer should not be considered the only barrier. Previous conclusions which were drawn from experiments using EDTA either to treat whole cells (8,10) or to prepare spheroplasts (5,11,15), and which suggested that the entry of actinomycin-D was prevented by the outer cell wall only, therefore required reconsideration and, possibly, alteration.

Studies on the effect of antibiotics which inhibit protein synthesis on the synthesis of APase, yielded results similar to those observed in the growth study. Streptomycin and chloramphenicol partially inhibit growth at 37°C and also inhibit APase synthesis. Although the rate of growth at 37°C is somewhat reduced in the presence of these antibiotics, the inhibition of APase synthesis is transitory and recovers after 30 min. Although growth is initially inhibited by these two agents at 46°C, the rate returns to normal within one hour.

The results collectively suggest that during growth at 46°C, the permeability of the outer cell wall is selectively altered so that mucopeptide synthesis-inhibiting antibiotics (such as ampicillin) become effective and that at this temperature some antibiotics, which inhibit protein synthesis (such as chloramphenicol) have increased access to their site of action. At higher temperature the permeability of the cytoplasmic membrane is not altered sufficiently to permit the penetration of actinomycin-D, an inhibitor of DNA dependent RNA synthesis.

Cultures grown at 46°C have altered outer wall permeability properties and these cultures also possess 40% less LPS in the outer cell wall structure than do those grown at 37°C. The cultures grown at 46°C produce more sedimentable LPS as compared to the cultures grown at 37°C indicating that the molecular weight of the LPS aggregates is larger in the former case. These data are interpreted to suggest that the high growth temperature decreases the association of lipopolysaccharide aggregates with the subsequent liberation of these fractions into the growth medium. Indeed, temperature

induced liberation of LPS has previously been observed in *P. aeruginosa* (16). The removal of these LPS fractions increases the permeability of the outer cell wall to certain antibiotics in a manner similar to the magnesium-facilitated permeability of the outer wall to lysozyme (2). Whether this permeability change is due entirely to the decreased amount of LPS or to some protein which may be complexed with LPS, such as found with APase-LPS (6), is under investigation at the present time.

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Table I

Susceptibility of *Pseudomonas aeruginosa* to Various Antibiotics

ANTIBIOTIC	GROWTH TEMPERATURE			
	37°C		46°C	
	INCUBATION TEMPERATURE			
	37°C	46°C	37°C	46°C
Chlortetracycline (10 µg)	11	14	14 ^a	18
Erythromycin (5 µg)	—	—	H	H
Chloramphenicol (10 µg)	—	14 ^a	11 ^a	25 ^a
Streptomycin (2 µg)	—	12	H	16 ^a
Tetracycline (10 µg)	9	25	10 ^a	30
Kanamycin (10 µ)	—	9	H	9
Lincomycin (2 µg)	—	—	H	H
Polymyxin-B (50 units)	10	10	12 ^a	12 ^a
Penicillin-G (5 units)	—	—	H	H
Ampicillin (2 µg)	—	—	H	H

a Secondary resistant colonies.

H A halo around disc; — no inhibition.

Figures given in the columns are the diameters of the zone of inhibition (in mm) around the discs.

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Table II

**The effect of growth temperature on the lipopolysaccharide
content of *Pseudomonas aeruginosa***

GROWTH TEMPERATURE	LIPOPOLYSACCHARIDE ($\mu\text{g}/\text{mg}$ of cell protein)
37°C	3.00
46°C	1.80

Cultures were grown at 37 and 46°C for 12 h and extracted with 45% phenol. LPS, as KDO containing material, was determined as described in Materials and Methods.

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Table III

**Lipopolysaccharide content of dialyzed cell-free culture
fractions of *Pseudomonas aeruginosa* grown at 37 and 46°C**

FRACTION	LIPOPOLYSACCHARIDE ($\mu\text{g}/\text{mg}$ of cell protein)	
	37°C	46°C
Supernatant Fluid	0.162	0.087
Pellet	0.209	0.347

Cell free supernatants were dialyzed against distilled water for 72 h and centrifuged at $47,000 \times g$ for 1 h. The fluids were removed and the pellets resuspended into 2 mL of distilled water. The fractions were assayed for protein and KDO as described in Materials and Methods.

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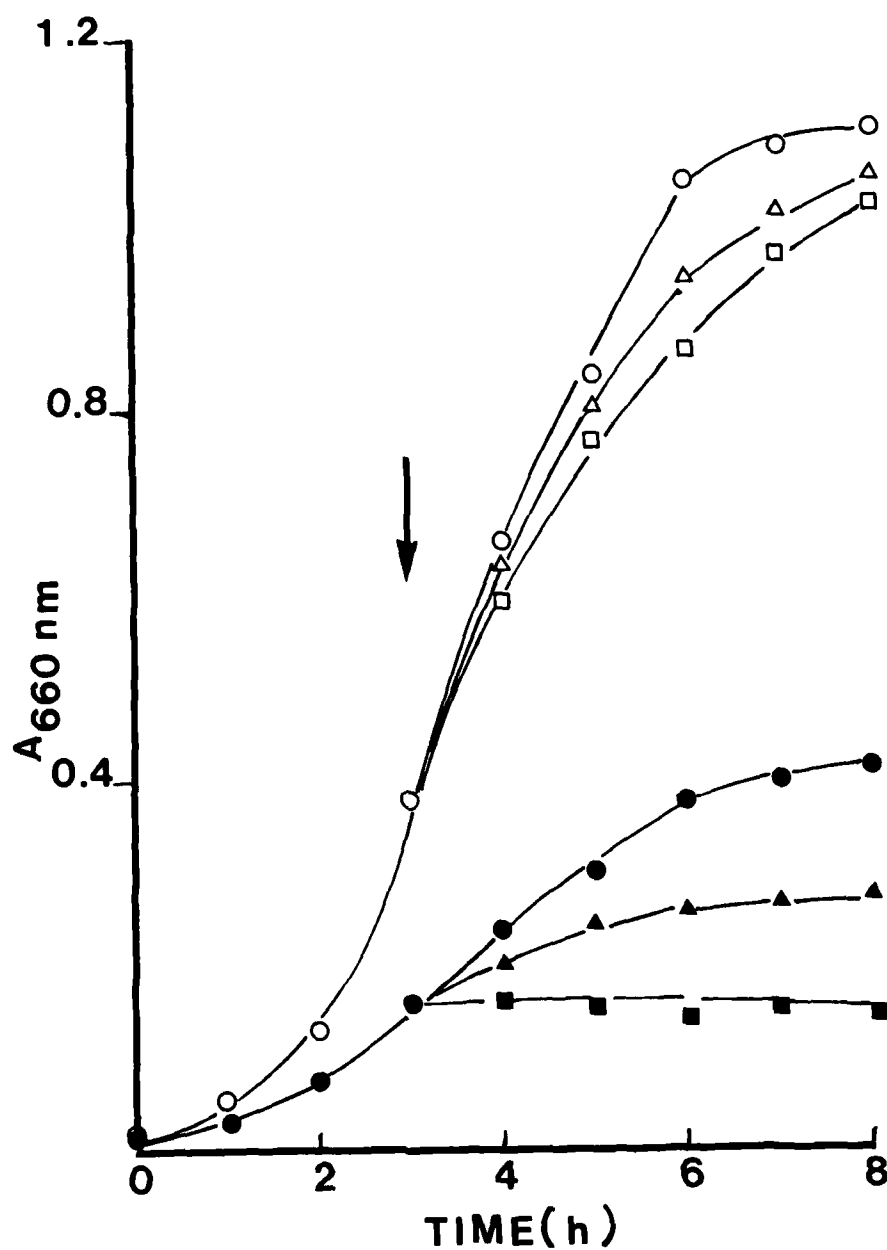


Figure 1

The effect of penicillin-G on the growth of *P. aeruginosa* grown at 37 and 46°C. Hollow and solid symbols represent growth at 37 and 46°C, respectively. Triangles and squares represent cultures containing 25 and 100 µg/mL of penicillin-G, respectively. The arrow indicates the time of addition of antibiotic.

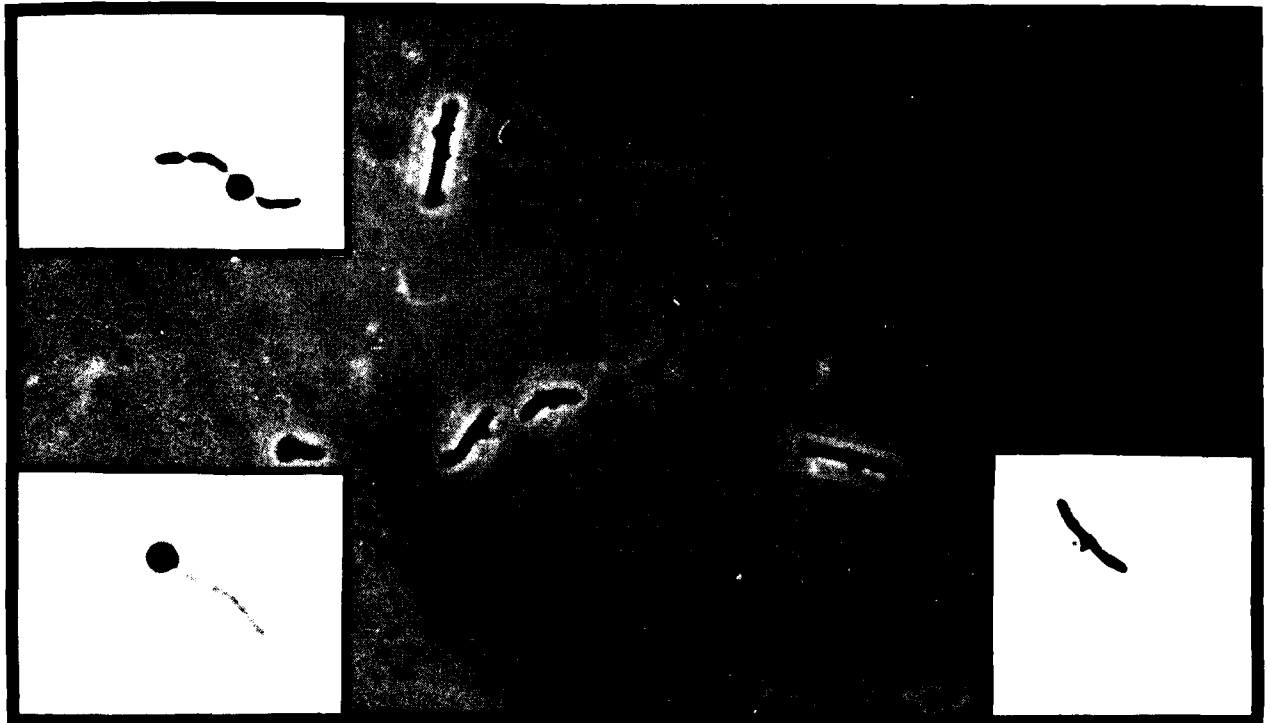


Figure 2

Phase contrast micrograph of filaments of *P. aeruginosa* which were grown at 46°C, treated with 100 µg/mL of penicillin-G and temperature shifted to 37°C.

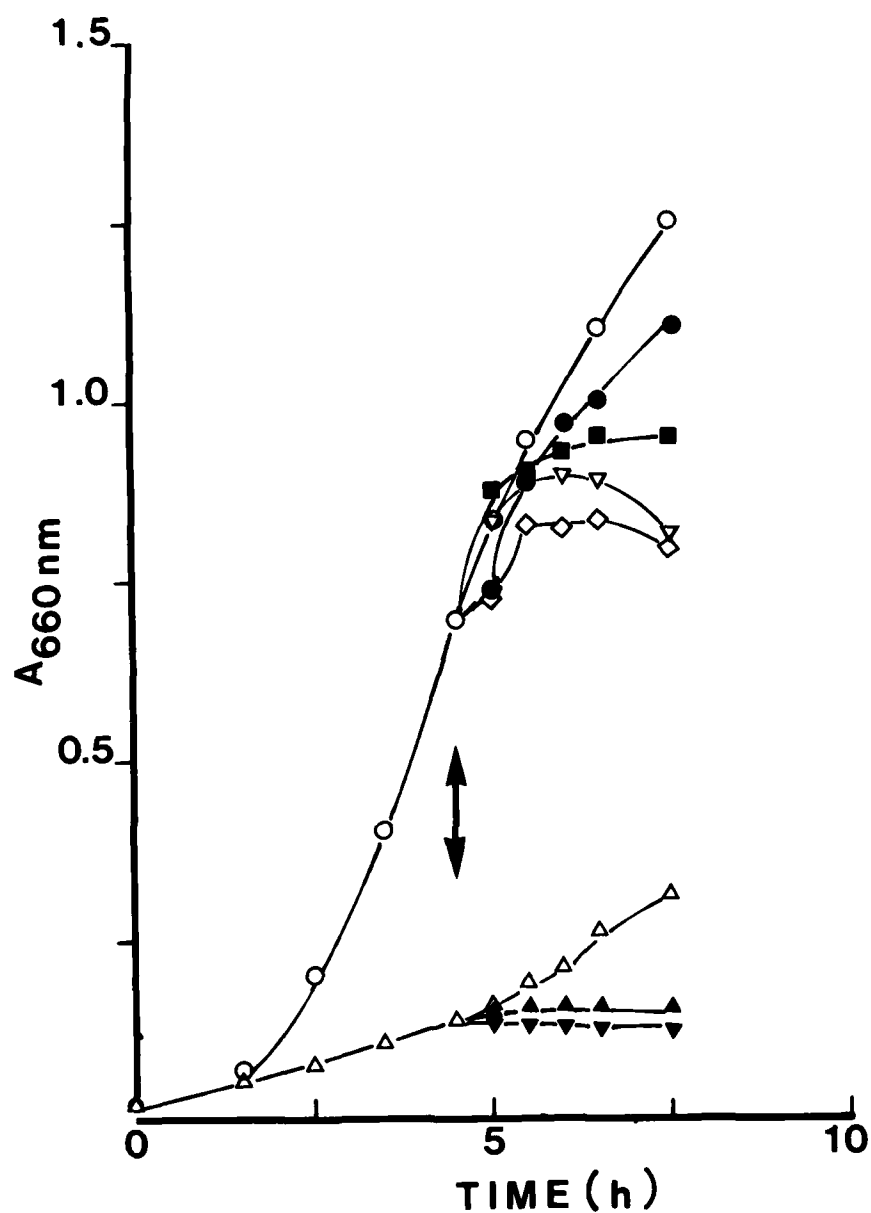


Figure 3

The effect of streptomycin on the growth of *P. aeruginosa* at 37 and 46°C. At the indicated time (an arrow), cultures grown at 37 and 46°C were divided equally and aseptically into five and three parts, respectively. To four flasks at 37°C were added 25 (●), 50 (■), 75 (▽) and 125 (◇) µg/mL streptomycin. To the cultures grown at 46°C were added 25 (▲) and 125 (▼) µg/mL of streptomycin. Hollow circles (○) and triangles (△) represent controls at 37 and 46°C, respectively.

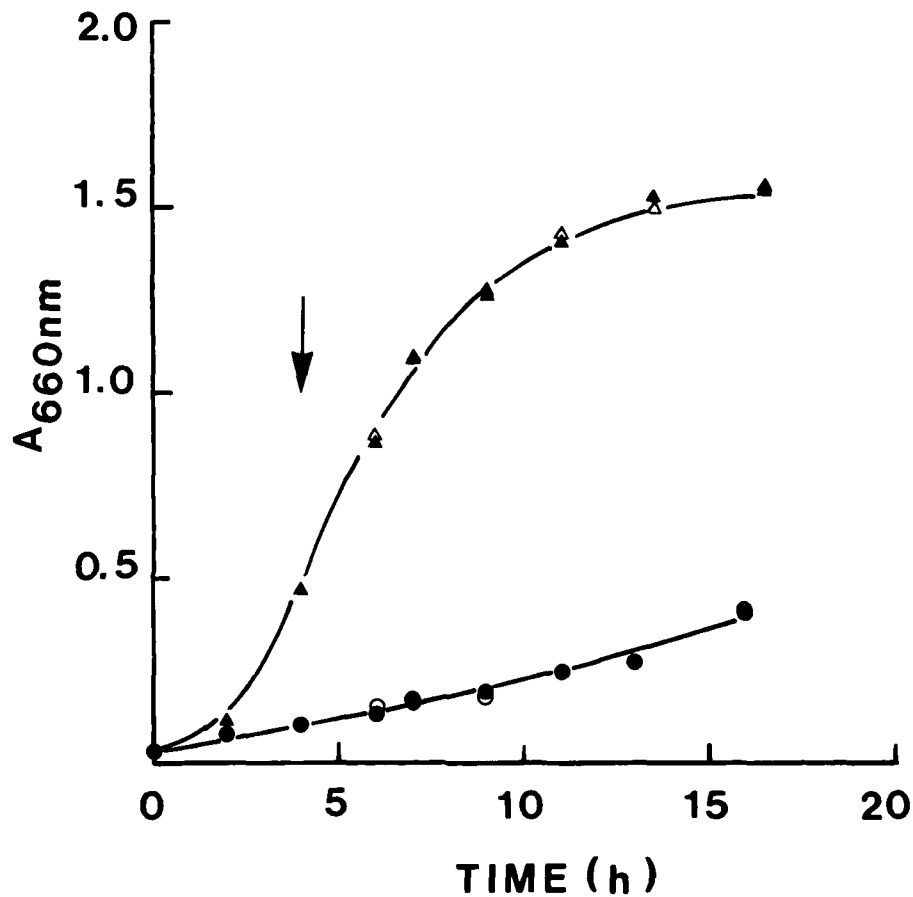


Figure 4

The effect of actinomycin-D on the growth of *P. aeruginosa* at 37°C and 46°C. Cultures were grown at 37°C (Δ, ▲) and 46°C (○, ●) and at the indicated time (arrow), both cultures were divided equally and aseptically into two parts. To one set of flasks at each temperature, was added actinomycin-D to give a final concentration of 10 μg/mL. Hollow and solid symbols represent growth in the presence and absence of antibiotic.

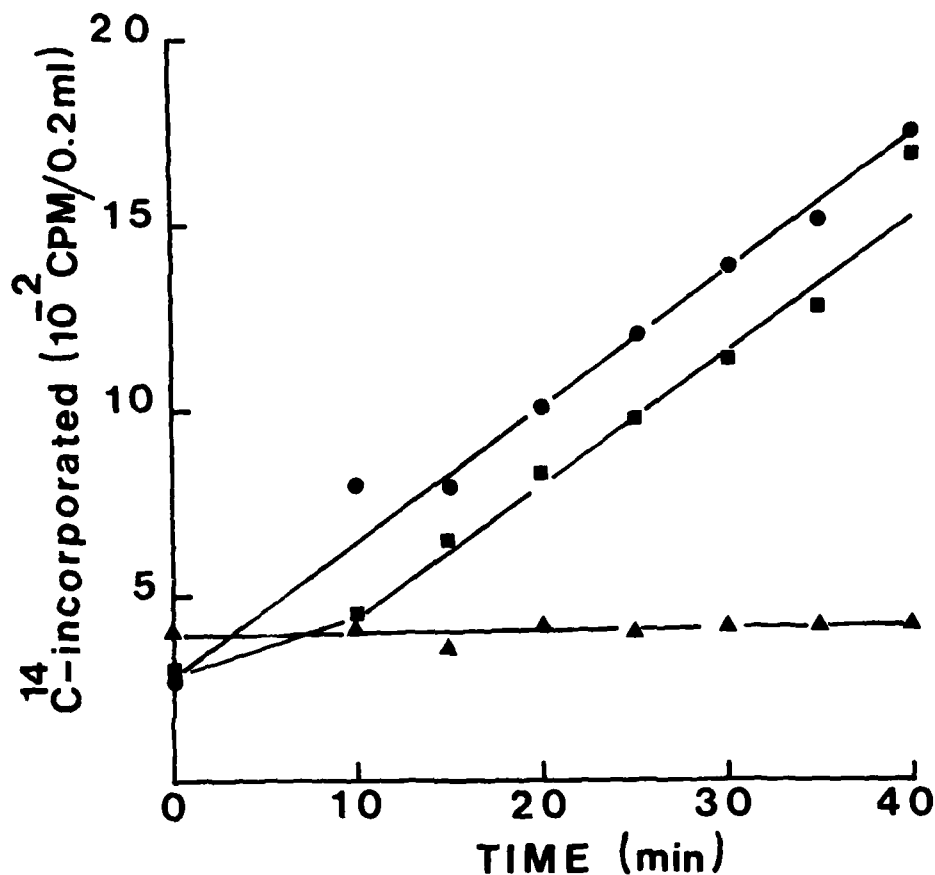


Figure 5

The effect of actinomycin-D and EDTA on the incorporation of uracil-2- ^{14}C into RNA of magnesium-lysozyme spheroplasts of *P. aeruginosa*. Spheroplasts of cultures grown at 46°C were suspended into growth medium to an optical density of 1.0. The incorporation of uracil-2- ^{14}C was performed at 46°C as described in Materials and Methods. Symbols used are: (●) spheroplasts, (■) spheroplasts and actinomycin-D ($20 \mu\text{g}/\text{mL}$), and (▲) spheroplasts with actinomycin-D ($20 \mu\text{g}/\text{mL}$) and EDTA (10 mM).

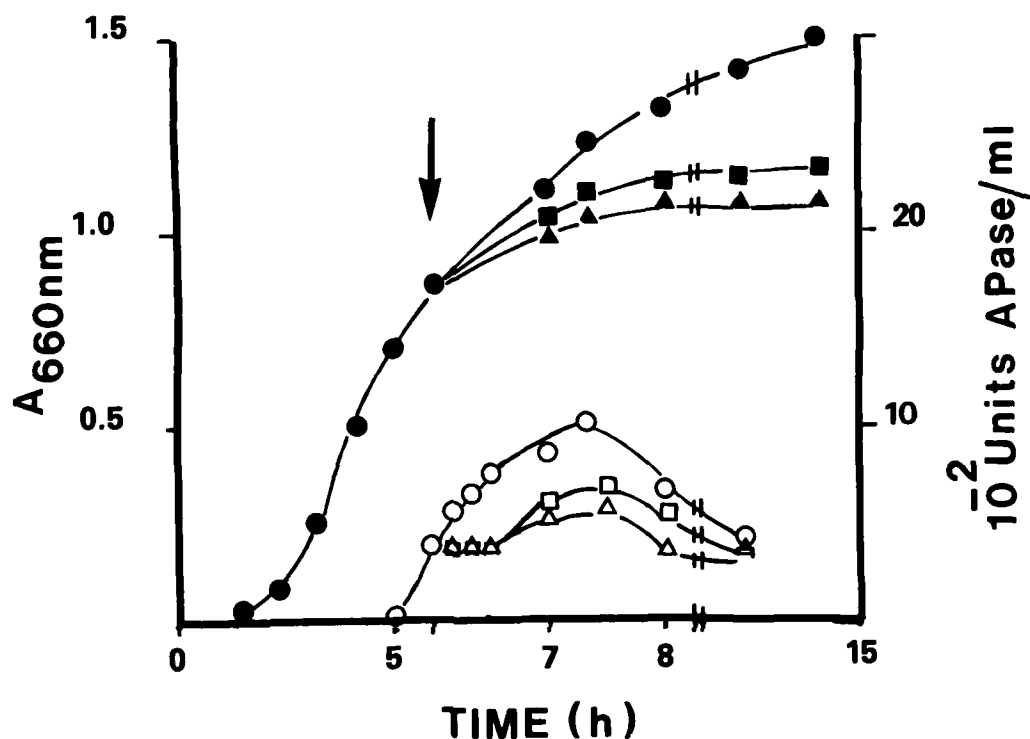


Figure 6

The effect of streptomycin and chloramphenicol on alkaline phosphatase synthesis at 37°C. The culture was grown at 37°C and after 6 h divided aseptically into three portions. Chloramphenicol (25 µg/mL) was added to one culture, to a second, streptomycin (125 µg/mL) and no addition was made to the third. At the indicated time intervals samples were withdrawn, analyzed for absorbance at 660 nm and APase activity in the cells as described in Materials and Methods. The solid symbols represent growth in the absence (●) and presence of streptomycin (▲) and Cm (■). The hollow symbols represent APase activities in the cells obtained from the respective cultures.

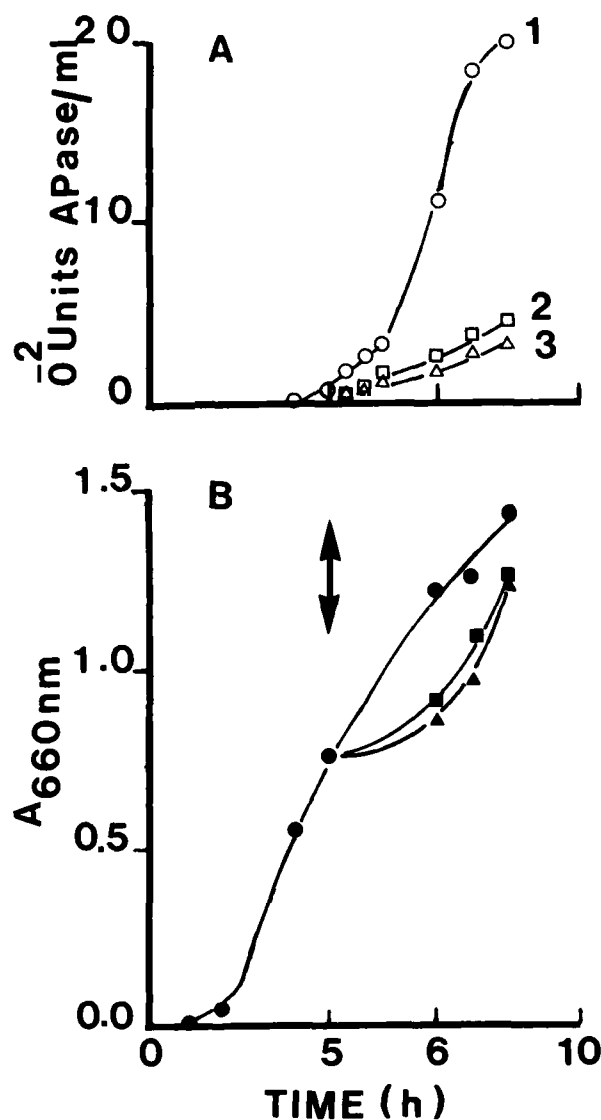


Figure 7

The effect of EDTA and actinomycin-D on APase synthesis at 37°C. Growth conditions were as described in Figure 5. At the indicated time (5 h), the culture was divided aseptically into three parts. To one was added EDTA (0.5 mM), to the second was added EDTA + actinomycin-D (0.5 mM + 10 µg/mL) and the third served as a control. At the indicated times samples were withdrawn and analyzed for (A) APase activity in control (○), EDTA (□) and EDTA + actinomycin-D (Δ) containing cultures. (B) growth of control (●), EDTA (■) and EDTA + actinomycin-D (▲) treated cultures.

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13. ABSTRACT <p><u>Pseudomonas aeruginosa</u>, which is resistant to a wide variety of antibiotics, becomes sensitive to several of these antibiotics when grown and tested at 46°C. Cell wall antibiotics such as penicillin-G and ampicillin are only effective when added to cells grown at 46°C prior to a temperature shift to 37°C. Antibiotics which must penetrate the cytoplasmic membrane to express their inhibiting action present a pattern different than those which are active against the outer cell wall. In order that these compounds be effective, the permeability of the cytoplasmic membrane must be further altered with agents such as EDTA which allows the penetration of actinomycin-D. Inhibitors of protein synthesis, streptomycin and chloramphenicol, have increased access to their sites of action in cells grown at 46°C. Cells grown at 46°C have 40% less lipopolysaccharide than cells grown at 37°C and the lipopolysaccharide aggregates are of large molecular size in cells grown at 46°C. It is concluded that growth at 46°C affects the permeability properties of the outer cell wall more than the permeability properties of the cytoplasmic membrane and that this is due, in part, to the selective release of lipopolysaccharide or lipopolysaccharide-protein complexes at elevated growth temperatures.</p>		

KEY WORDS

Pseudomonas aeruginosa, antibiotics, sensitivity, spheroplast, lipopolysaccharide, cell division, alkaline phosphatase

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